Bull. Environ. Contam. Toxicol. (1985) 34:668-675

Bulletin of Environmental Contamination and Toxicology

Effects of Storage and Pesticide Treatments on Honey Bee Brain Acetyl Cholinesterase Activities

G. E. Westlake,[1] A. R. Hardy,[1] and J. H. Stevenson[2]

[1]Tolworth Laboratory, Agricultural Science Service, Ministry of Agriculture, Fisheries and Food, Surbiton, Surrey KT6 7NF, United Kingdom and
[2]Rothamsted Experimental Station, Harpenden, Herts. AL5 2JQ, United Kingdom

The poisoning of honey bees *(Apis mellifera)* arising from the agricultural use of pesticides has been monitored in the United Kingdom for over twenty years (Stevenson *et al.* 1978) using measurement of bee head acetylcholinesterase (AchE) inhibition as an indicator of organophosphate insecticide poisoning. In 1978, there were widespread reports of serious poisoning associated with honey bees foraging on oilseed rape to which triazophos had been applied. Investigation of these incidents showed that AchE inhibition was apparently reversible after triazophos poisoning (Findlay *et al.* 1982).

The present study was undertaken to establish whether measurement of head AchE can be used to diagnose pesticide poisoning in honey bees. This paper describes experiments to examine enzyme stability and the degree and reversibility of inhibition following lethal exposure to a range of pesticides. The study has shown bee AchE to be unstable when stored at room temperature. Lethal pesticide treatment of bees with most carbamate and organophosphate compounds resulted in significant AchE inhibition, which was not spontaneously reversible following storage at room temperature, but reactivation could be achieved by chemical means. Oral treatment with carbaryl and pirimicarb did not result in the inhibition of honey bee head AchE. Although pyrethroids and gamma-HCH treatments did not inhibit bee AchE, the enzyme lost activity on storage at room temperature. This lost activity was recovered following chemical reactivation. The results of this study will allow the interpretation of bee incidents where pesticide poisoning is alleged.

MATERIALS AND METHODS

The insecticides selected for study were : *Organophosphate* - azinphos methyl /O,O-dimethyl S-/(4-oxo-1,2,3-benzotriazin-3 (4H)-yl)methyl/ phosphorodithioate/; demeton-S-methyl /S-2-ethyl-thioethyl O,O-dimethyl phosphorothioate/; dimethoate /O,O-dimethyl S-/2-(methylamino)-2-oxoethyl/ phosphorodithioate/; phosalone

/S-/6-chloro-2-oxo-3(2H)-benzoxazolyl/ methyl/O,O-diethyl phosphorodithioate/; triazophos /O,O-diethyl O-(1-phenyl-1 H-1, 2,4-triazol-3-yl) phosphorodithioate/; *Carbamate* - carbaryl /1-naphthalenyl methylcarbamate/; ethiofencarb /2-ethylthio-methylphenyl methylcarbamate/; pirimicarb /2-(dimethylamino)-5,6-dimethyl-4-pyrimidinyl dimethylcarbamate/; *Pyrethroid* - cypermethrin /(RS)-cyano(3-phenoxyphenyl)methyl(IRS)-*cis,trans*-3-(2,2-dichloroethenyl)-2,2-dimethyl cyclopropane carboxylate/; deltamethrin /(IR/α(S*),3α/)-cyano(3-phenoxyphenyl)methyl 3-(2,2-dibromoethenyl)-2,2-dimethyl-cyclopropanecarboxylate/; permethrin /(3-phenoxyphenyl)methyl(IRS)-*cis,trans*-3-(2,2-dichloroethenyl)-2,2-dimethylcyclo-propane carboxylate/; *Organochlorine* - gamma-HCH /1α,2α,3β,4α,5α,6β-hexachlorocyclohexane/. The pesticides were all of technical grade. The pyrethroids were synthesised at Rothamsted Experimental Station.

Batches of worker honey bees *(Apis mellifera)* were kept 20 to a cage at 28°C. Untreated bees were killed by storing at -20°C for 24 hours. The effect of temperature on untreated bee head AchE activity was investigated by storage of bees at -20°C, 4°C and room temperature for periods of 1,4,7,12,18 and 26 days after death in unsealed glass bottles. Further individual bees were dosed (Anon, 1979) by contact with an amount of each pesticide calculated to be three times the LD_{50} (Smart and Stevenson, 1982, Stevenson, 1978). Bees were also dosed orally with pirimicarb because of the high contact LD_{50} value with honey bees and with carbaryl for comparison. All treated bees were left for 24 hours at 28°C. Dead bees were collected and the survivors killed along with batches of untreated bees. All bees were frozen at -20°C for 24 hours. Bees which had died from pesticide treatments and untreated bees were divided into groups for either immediate bee head AchE assay or for storage at -20°C or at room temperature for a further 7 days prior to AchE assay. Duplicate groups of 15 bee heads were cut from all untreated and treated groups of bees and homogenised in 10 ml ice cold 0.1M phosphate buffer pH 7.5 using a Silverson macerator. The homogenate was filtered through glass wool prior to assay and reactivation. The AchE activity of the extract was assayed at pH 7.5 by the method of Ellman *et al.* (1961) modified using a 1 ml of 20% (v/v) extract, 1.9 ml of 0.0015M DTNB in 0.1M phosphate buffer and 0.1 ml of 0.02 M ATCl in a final volume of 3.0 ml at 25°C and 412 nm.

Chemical reactivation was initiated by mixing 0.2 ml of extract and 0.05 µl of 3.3% (v/v) aqueous pyridine 2-aldoxime methiodide (2-PAM) solution. Samples (100 µl) were taken for assay after 30 minutes. The method described by Martin *et al.* (1981) for spontaneous reactivation of the AchE activity was also applied to the bee head extracts.

Table 1. Brain AchE activity[a] in bees[b] kept at -20°C, 4°C and room temperature for periods of up to 26 days after sacrifice.

Period of storage (days)	-20°C		4°C		Room temperature	
	Activity	Activity after chemical reactivation	Activity	Activity after chemical reactivation	Activity	Activity after chemical reactivation
1	100	76	nm	nm	nm	nm
4	89	nm	124	nm	110	nm
7	124	119	133	112	116	114
12	77	71	89	76	73	62
18	97	87	115	90	62	61
26	95	81	97	80	9*	50*

a. Expressed as percentage of mean control activity (119 I mU/bee head)
b. Duplicate samples of 15 bees/group
nm not measured
Significant difference from mean control activity on day 1
*p <0.001, Student's t test

RESULTS AND DISCUSSION

Storage conditions. Untreated bees were stored at room temperature in unsealed glass bottles to prevent both condensation forming with the subsequent appearance of mould and the general deterioration of the samples. A loss of body water, which amounted to approximately 60% of the initial bee head weights, occurred during storage at room temperature for 7 days and meant that neither the bee nor bee head weight could be used to express bee head AchE activities. These have therefore been expressed in International milliunits (mmoles of substrate hydrolysed per minute) of enzyme activity per bee head and this has given reproducible results.

Storage of untreated bee heads at $-20^{o}C$ or $4^{o}C$ for periods of up to 26 days resulted in no significant loss of AchE activity although some variation was found compared to the initial activity on day 1 (Table 1). The chemical reactivation procedure applied to these bee head extracts caused some small decreases in activities. In contrast, bees stored at room temperature showed appreciable losses in head AchE activity which became significant after 26 days. Some degree of chemical reactivation was also evident at 26 days.

Table 2. Contact and oral LD_{50} values for the honey bee and treatments at 2 or 3 x LD_{50} levels

Compound	LD_{50} µg contact	Dose µg/bee	Compound	LD_{50} µg contact	Dose µg/bee
Azinphos-methyl	0.063	0.15	Cypermethrin	0.056	0.12
Demeton-S-methyl	0.26	0.60	Deltamethrin	0.051	0.11
Dimethoate	0.12	0.30	Permethrin	0.11	0.25
Phosalone	8.9	20	HCH	0.46	1.2
Triazophos	0.055	0.15			
Carbaryl	1.3	2.7		LD_{50}	
Ethiofencarb	2.3	6.3	Compound	µg oral	Dose µg/bee
Pirimicarb	>54	140	Carbaryl	0.14	0.28
			Pirimicarb	3.2	6.5

Table 3. Brain AchE activity[a] in bees following 2 or 3 x LD_{50} pesticide treatments kept at -20°C for 1 and 8 days after death and at -20°C for 1 day after death followed by 7 days at room temperature.

Compound	-20°C for 1 day		-20°C for 8 days		-20°C for 1 day then room temperature for 7 days	
	Activity	Activity after chemical reactivation	Activity	Activity after chemical reactivation	Activity	Activity after chemical reactivation
Control	100[h]	89[i]	117	113[fj]	110	108[fj]
Azinphos-methyl	53[cj]	71[f]	52[j]	65[fj]	25[j]	53[f]
Demeton-S-methyl	19[c]	76[f]	16	56[e]	4[d]	55[f]
Dimethoate	17[c]	77[e]	14	56[f]	6	53[f]
Phosalone	13[c]	83[f]	16	71[f]	15	58[f]
Triazophos	71[b]	94[fh]	64[h]	72[fh]	41[dg]	63[e]
Carbaryl	22[cg]	68[e]	21[k]	59[k]	7[dg]	49[fg]
Ethiofencarb	53[c]	73[e]	62[k]	69[k]	29[d]	53[e]
Pirimicarb	40[c]	66[f]	39[k]	52[k]	14[d]	54[f]
Cypermethrin	84	nm	80	96	33[d]	79[f]
Deltamethrin	98	nm	72	94	39[d]	81[f]
Permethrin	87	nm	79	95	40[dj]	82[ej]
HCH	88[j]	nm	86[j]	87[j]	51[dj]	72[ej]
Carbaryl (oral)	82	77	nm	nm	nm	nm
Pirimicarb (oral)	98	96	75	71	80	81

a. expressed as percentage of mean control activity (126 I mU/bee head)
b. inhibition significant at $p < 0.05$
c. inhibition significant at $p < 0.005$
d. activity change significant at $p < 0.05$
e. reactivation significant at $p < 0.05$
f. reactivation significant at $p < 0.005$

Duplicate samples of 15 bees except for
g. 8 groups of 15 bees
h. 6 groups of 15 bees
i. 3 groups of 15 bees
j. 4 groups of 15 bees
k. single group of 15 bees
nm - not measured

Contact treatments. The majority of bees receiving pesticide treatments (Table 2) died within 24h. Bees poisoned by contact application of organophosphate and carbamate insecticides gave significant AchE inhibition after death and storage at -20°C for one day, but those treated with pyrethroids and gamma-HCH did not (Table 3). Phosalone, dimethoate and demeton-S-methyl gave 13-19% of the control activity, azinphos-methyl and triazophos gave 53 and 71% of the control activity respectively and the three carbamates (carbaryl, ethiofencarb and pirimicarb) gave 22-53% of the control activity. The present results with dimethoate are in agreement with those of Needham *et al.* (1966) who reported 10% of the control activity for bees examined one day after dimethoate poisoning. However, the results with triazophos differ from those of Findlay *et al.* (1982) who found approximately 8% of control activity in bees two days after triazophos poisoning. This spontaneously reactivated to 44% of control activity after 7 days at room temperature. In the current study, the initial activity obtained one day after poisoning was 71% of the control value. Needham *et al.* (1966) and Stevenson *et al.* (1978) have previously suggested that honey bee cholinesterase activities below 33% of the control indicate probable poisoning by organophosphate pesticides while Barker *et al.* (1978) stated that intermediate AchE levels of 33-67% of the control indicates possible organophosphate poisoning. The current study and that of Findlay *et al.* (1982) show the latter criteria are too rigid since bees poisoned with azinphos-methyl, triazophos and the carbamate ethiofencarb all show 53-71% of the control activities.

The inhibition from both organophosphate and carbamate pesticides was largely recoverable to normal levels by chemical reactivation with 2-PAM although the amount of activity recovered varies between the compounds. Martin *et al.* (1981) also obtained chemical reactivation of quail brain AchE with organophosphate and carbamate pesticides, however, the differential reactivation for dimethyl and diethyl organophosphorus esters was not evident in the present study with bee AchE. The method described by Martin *et al.* (1981) for the differential reactivation of quail brain AchE resulting from organophosphate and carbamate pesticides using chemical and spontaneous reactivation procedures respectively has been routinely used at the Tolworth Laboratory for the investigation of wildlife deaths of avian and mammalian species in which agricultural chemicals are implicated (Brown *et al.* 1977). However, no AchE activity from bee head homogenates was detectable following elution through Sephadex columns with or without further dilution thereby preventing the routine application of this differential procedure to the investigation of pesticide effects in bee poisoning incidents.

The bee head AchE activities from treated bees following storage at -20°C for 8 days all appeared to be in similar ranges to the initial depressions obtained after storage at -20°C for 1 day. The subsequent response of the inhibited AchE to chemical reactivation was reduced following storage at -20°C. With the exception of phosalone, further large reductions in bee head AchE activity

were recorded after storage at room temperature for 7 days and again underlines the need for caution in interpreting AchE activity data obtained from bee incident samples when the time following death is unknown. The three pyrethroids and gamma-HCH did not depress bee head AchE activities following storage at -20°C. However, bees treated with these pesticides and stored at room temperature lost 49 to 67% of their activities but could be chemically reactivated to higher levels than were obtainable with any of the organophosphate or carbamate contact treatments. The mechanism responsible for the latter changes following storage after pyrethroid and gamma-HCH treatments are not understood.

Oral treatments. Bees which died from oral carbaryl or pirimicarb treatments did not show significant inhibition of AchE activity (Table 3). Storage of bees treated with orally dosed pirimicarb for 7 days at room temperature did not result in significant decreases in the AchE activity and no subsequent chemical reactivation was obtained.

Inhibition of cholinesterase by carbamates has previously been assumed by Stevenson *et al.* (1978) to be rapidly reversible after death and the current results are consistent with this view for oral administration of carbaryl and pirimicarb. The results for oral carbaryl and pirimicarb are in contrast to the results from the contact treatments with carbaryl, ethiofencarb and pirimicarb which all give significant inhibition which is also persistent on storage at room temperature for 7 days. This suggests that following oral treatment, carbaryl and pirimicarb are either detoxified metabolically or spontaneously decompose in the gastrointestinal tract while that applied externally would either be absorbed directly into all tissues or remain on the exterior surface and react with the enzyme during the assay.

Interpretation. The results demonstrate that the application of head AchE assays to the diagnosis of organophosphate or carbamate insecticide poisoning in bees requires a knowledge of the exact storage history of the samples. In many mortality incidents the casualties are not discovered until some time after death with the result that the insects have spent a variable time at relatively high temperatures before reaching the laboratory for analysis. In such cases the AchE results require cautious interpretation to avoid any loss of activity due to deterioration during delay periods being incorrectly attributed to pesticide involvement.

An integral part of the routine investigation of suspected bee poisoning incidents is a field investigation to determine the scale of reported mortalities, the proximity of attractive crops and any evidence of recent pesticide treatments. The collected bee samples are routinely screened in order to eliminate disease as the probable cause of death. If these tests are negative, bee head AchE is measured followed by analysis for organophosphorus, organochlorine and carbamate pesticide residues. On the basis of the present study, the measurement of the relative

activities determined before and after chemical reactivation of bee head AchE is useful in differentiating between low esterase values which are due to ageing and deterioration of bee samples, before collection and during transit to the laboratory, and low values due to pesticide exposure. This esterase measurement should only be used as an adjunct to residue analysis but may be beneficial in the final causal analysis of an incident if very low levels of pesticide residues are present in the bee samples extracted.

REFERENCES

Anon (1979). UK Pesticides Safety Precautions Scheme, Working Document D3, Laboratory testing for toxicity to honey bees. Ministry of Agriculture, Fisheries and Food, London.

Barker RJ, Lehner Y, Kunzmann MR (1978). The constancy of cholinesterase activity in adult honey bees. J.Apic.Res. 17 173-175.

Brown PM, Bunyan PJ, Stanley PI (1977). The investigation and pattern of occurrence of animal poisoning resulting from the misuse of agricultural chemicals. J.Forens.Sci.Soc. 17 211-221.

Ellman GL, Courtney KD, Andres V, Featherstone RM (1961). A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem. Pharmacol. 7 88-95.

Findlay E, Smart LE, Stevenson JH (1982). Identification of poisoning by triazophos in honey bees. J.Apic.Res. 21 226-231.

Martin AD, Norman G, Stanley PI, Westlake GE (1981). Use of reactivation techniques for the differential diagnosis of organophosphorus and carbamate pesticide poisoning in birds. Bull.Environ.Contam.Toxicol. 26 775-780.

Needham PH, Solly SRB, Stevenson JH (1966). Damage to honey bee colonies *(Apis mellifera)* by insecticides in Great Britain, 1956-63. J. Sci. Fd. Agric. 17 133-137.

Smart LE, Stevenson JH (1982). Laboratory estimation of toxicity of pyrethroid insecticides to honey bees : relevance to hazard in the field. Bee World 63 150-153.

Stevenson JH (1978). The acute toxicity of unformulated pesticides to worker honey bees *(Apis mellifera L.)*. Plant Pathology 27 38-40.

Stevenson JH, Needham PH, Walker J (1978). Poisoning of honey bees by pesticides : Investigations of the changing pattern in Britain over 20 years. Rothamsted Experimental Station Report for 1977 Part 2, 55-72.

Received April 23, 1984; accepted June 18, 1984